Setup and transfer of a gradient HILIC method for the impurity analysis of temozolomide

From an Agilent 1260 Infinity LC system to a Vanquish Core HPLC system

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Application benefits

- A fast and reproducible gradient HILIC method was set up for the impurity analysis of the hydrophilic temozolomide.
- Straightforward transfer of the method from an Agilent™ 1260 Infinity™ LC system to a Thermo Scientific™ Vanquish™ Core HPLC system was demonstrated.
- Equivalent chromatographic results were obtained.

Goal

Develop an analytical HILIC method and transfer it from an Agilent 1260 Infinity LC system to the Vanquish Core HPLC system.

Introduction

The transfer of liquid chromatographic (LC) methods from one instrument to another is a recurring task in many analytical laboratories. It is required, for example, when a high workload has to be distributed over several instruments, when legacy instrumentation is replaced by modern technology, or when method development is realized in a lab different than the lab for the final method implementation, such as when outsourcing to contract labs. In each case, the transfer is only considered effective if equivalent results are obtained by the sending and receiving unit, but the challenge and effort depend on multiple factors. Usually a transfer is straightforward if equal instruments are involved, but deviations in technical characteristics of the systems like gradient delay volumes (GDV), pump mixing modes, column thermostatting and eluent pre-heating options, etc. may affect critical

results like peak resolution or retention times.[1] In that, the complexity of the transfer job depends on the requirements of the chromatographer to the analytical outcome and the defined limits of acceptable deviations from the originating system. Usually very limited modifications of method parameters are acceptable during a transfer to prevent the need of a time-consuming revalidation. Hardware and software features of the Vanquish Core HPLC system are designed to assist in transferring LC methods while adhering to regulatory guidelines.[2,3]

Reversed-phase (RP) is the most common LC technique and it is widely used in pharmaceutical small molecule analyses. The majority of pharmacopeial LC methods rely on RP separations. However, hydrophilic interaction liquid chromatography (HILIC) is gaining more and more interest due to its polar retentivity. As very polar molecules are poorly retained by RP or only under special conditions like tedious chemical derivatization of analytes or ion-pairing reagents in the mobile phase, HILIC consolidates in its role as a powerful alternative technique. One example is the hydrophilic temozolomide: an alkylating cytostatic drug used in the chemotherapy of brain tumor patients. The chromatographic method for the impurity analysis of temozolomide provided by the monographs[4,5] of the European and the United States Pharmacopeia (Ph. Eur. and USP) specifies a C18 stationary phase and a highly aqueous mobile phase with the ion-pairing reagent hexanesulfonate to separate the active pharmaceutical ingredient (API) and four related impurities. In the current work, an alternative HILIC method was set up and was transferred from an Agilent 1260 Infinity LC (1260 Infinity) system to a Vanquish Core HPLC system.

Experimental

Instrumentation

Instruments listed below were used in the current study.

Reagents and materials

- Deionized water, 18.2 MΩ·cm resistivity or higher
- Fisher Chemical™ Optima™ Acetonitrile LC/MS grade (P/N A955-212)
- Fisher Chemical™ Optima™ Ammonium acetate (NH_4Ac) LC/MS grade (P/N A114-50)
- Fisher Chemical™ Optima™ Acetic acid glacial (P/N A465-250)
- Fisher Chemical™ Hydrochloric acid 37% (P/N H/1200/PB08)
- Ph. Eur. reference standard: Temozolomide CRS batch 1, catalog code Y0001827[6]
- Ph. Eur. reference standard: Temozolomide for peak identification CRS batch 1, catalog code Y0001960[6]

Sample preparation

- Sample I: 5 mg of the reference standard temozolomide CRS were dissolved in 5 mL acetonitrile.
- Sample II: 3 mL of sample I were mixed with 3 mL of a 10.3 g/L aqueous hydrochloric acid solution. The mixture was heated in a boiling water bath for 1 hour in order to generate the impurities A, B, and E by forced degradation.
- Sample III: 1 mg of the reference standard temozolomide for peak identification CRS was dissolved in 1 mL acetonitrile. The standard contains the API temozolomide and low amount of impurity D.
- Sample IV: 450 µL of sample III were spiked with 15 µL of sample II to generate a sample of high API and low impurity concentration.

	Agilent 1260 Infinity Quaternary	Vanquish Core Quaternary
System base		System Base Vanquish Core (P/N VC-S01-A-02)
Pump	Quaternary pump (G1311B)	Quaternary Pump C (P/N VC-P20-A-01)
Sampler	High Performance Autosampler (G1367E) with thermostat module (G1330B)	Split Sampler CT (P/N VC-A12-A-02)
Column compartment	TCC with 6 µL heat exchanger (G1316A)	Column Compartment C (P/N VC-C10-A-03)
Detector	Variable Wavelength Detector (G1314F)	Variable Wavelength Detector C (P/N VC-D40-A-01)
Flow cell	Standard (10 mm, 14 µL, G1314-60186)	Standard (10 mm, 11 µL P/N 6077.0250)
System accessories		Method Transfer Kit Vanquish (P/N 6036.2100) Strong Solvent Loop (P/N 6036.2200)

HPLC conditions

Parameter	Value
Column	Thermo Scientific™ Syncronis™ HILIC, 4.6 × 100 mm, 3 µm, 175 Å (P/N 97503-104630)
Mobile phases	A: 20 mM NH_4Ac pH 5.1 in water B: 20 mM NH_4Ac pH 5.1 in water/ acetonitrile (10/90; v/v)
Flow rate	1 mL/min
Gradient	0.0 min – 100% B 5.0 min – 44% B 6.0 min – 44% B 6.1 min – 100% B 12 min – 100% B
Column temperature	30 °C (still air)
Autosampler temperature	10 °C
Detection	266 nm, 10 Hz, response time 1 s (1260 Infinity) / 0.5 s (Vanquish Core)
Injection volume	10 µL (5 µL for sample II)
Needle wash	Off

Experiments were generally verified by three consecutive injections, except for repeatability data, which was recorded with eight consecutive injections.

Data processing and software

Thermo Scientific™ Chromeleon™ Chromatography Data System (CDS) 7.3 was used for data acquisition and analysis.

Results and discussion

Method

The structures of the API temozolomide and the impurities A, B, D, and E, pursuant to the Ph. Eur. Nomenclature,[4] are summarized in Figure 1. The column selected for the separation was a Syncronis HILIC LC column with a proprietary zwitterionic stationary phase.

Figure 2 shows the chromatograms obtained by the described HILIC method with samples II, III, and IV with the 1260 Infinity LC system. In sample II, the majority of the API temozolomide was converted into the three impurities A, B, and E due to the forced degradation procedure in aqueous hydrochloric acid. Impurity D was detected as a small peak in sample III. Good separation of all peaks was ahieved, with a separation of impurities E and D to be expected from the chromatograms. To create a sample IV with all four impurities, sample III was spiked with a small amount of sample II. However, only three impurity peaks A, B, and D were detected with increased area of impurity D (compared to sample III) while no peak of impurity E was observed.

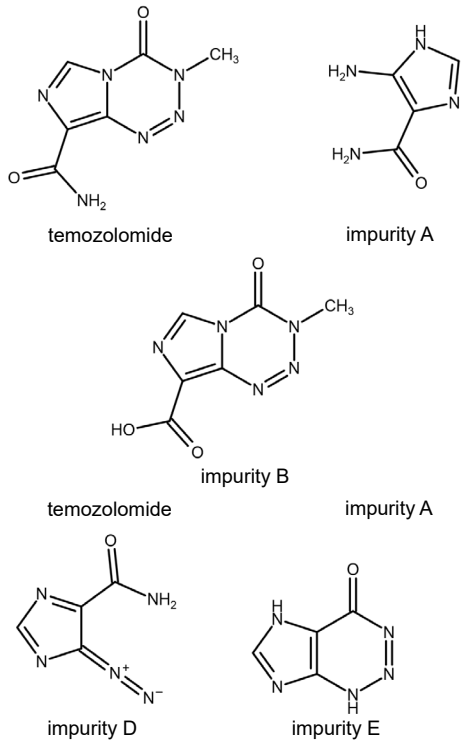

Figure 1. Structures of the API temozolomide and its impurities according to Ph. Eur.[4]

These and a few more experiments (data not shown) pointed to the assumption that impurity E transforms into impurity D by a ring opening under the much less acidic conditions in sample IV. No other sample preparation, mobile phase, etc. was found to create all four impurity peaks at the same time. Thus, injection of sample II and III is required for retention time comparison and impurity identification, which is in accordance with the Ph. Eur. procedure[4] for peak identification.

The gradient HILIC method was fast with a run time of 12 min and distinctly faster than the isocratic Ph. Eur. RP method, which can easily be around 30 min (specified as 3 times the retention time of temozolomide). As HILIC is commonly known to need long equilibration times, it is worth mentioning here that the equilibration time of 6 min was quite short, corresponding to just 5–6 column volumes. However, recent studies confirmed the validity of the “repeatable partial equilibration” concept in HILIC, proving that stable retention times can be achieved with not fully equilibrated columns.[7,8] Constant equilibration time was identified as a prerequisite, as selectivity changes may occur for different equilibration times. The effect is also shown in Figure 3 for the current method with additional equilibration time. As the resolution was impaired and no benefit was visible from longer equilibration times, the final method was kept as short as possible (6 min equilibration time).

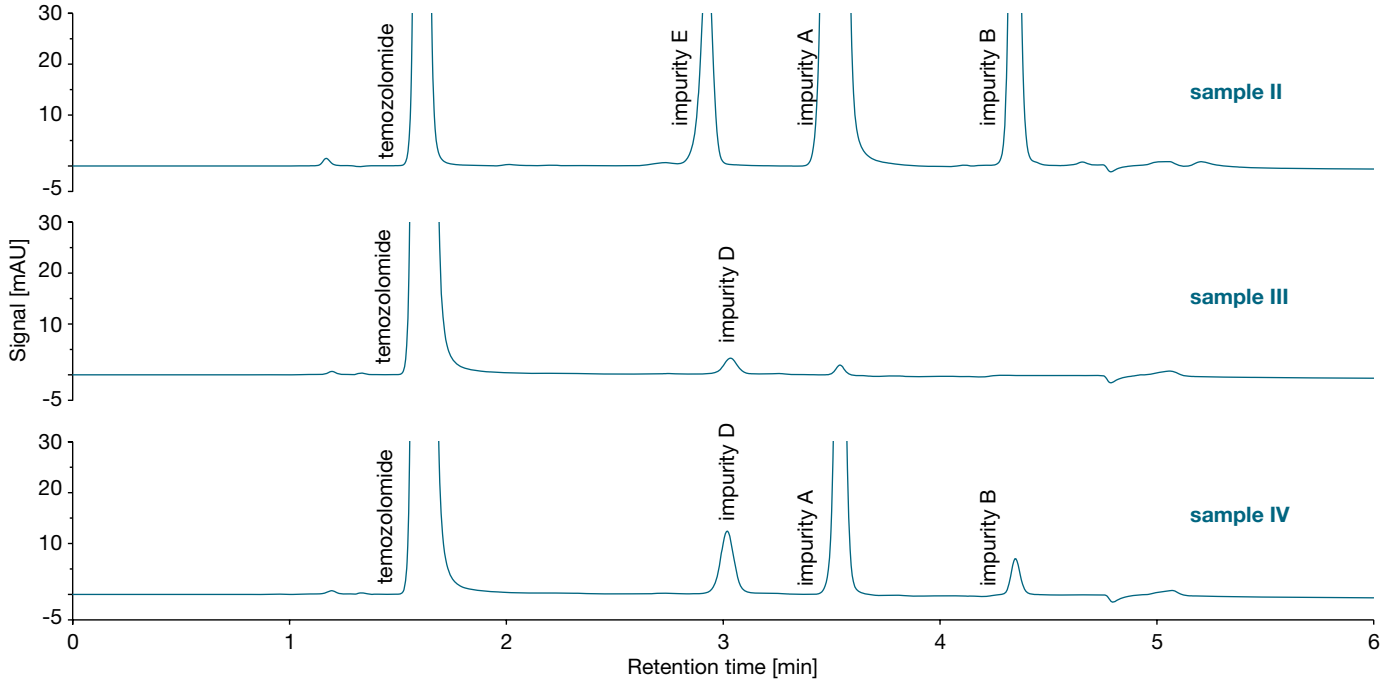

Figure 2. Chromatograms obtained by samples II, III, and IV with the 1260 Infinity system (6 min equilibration)

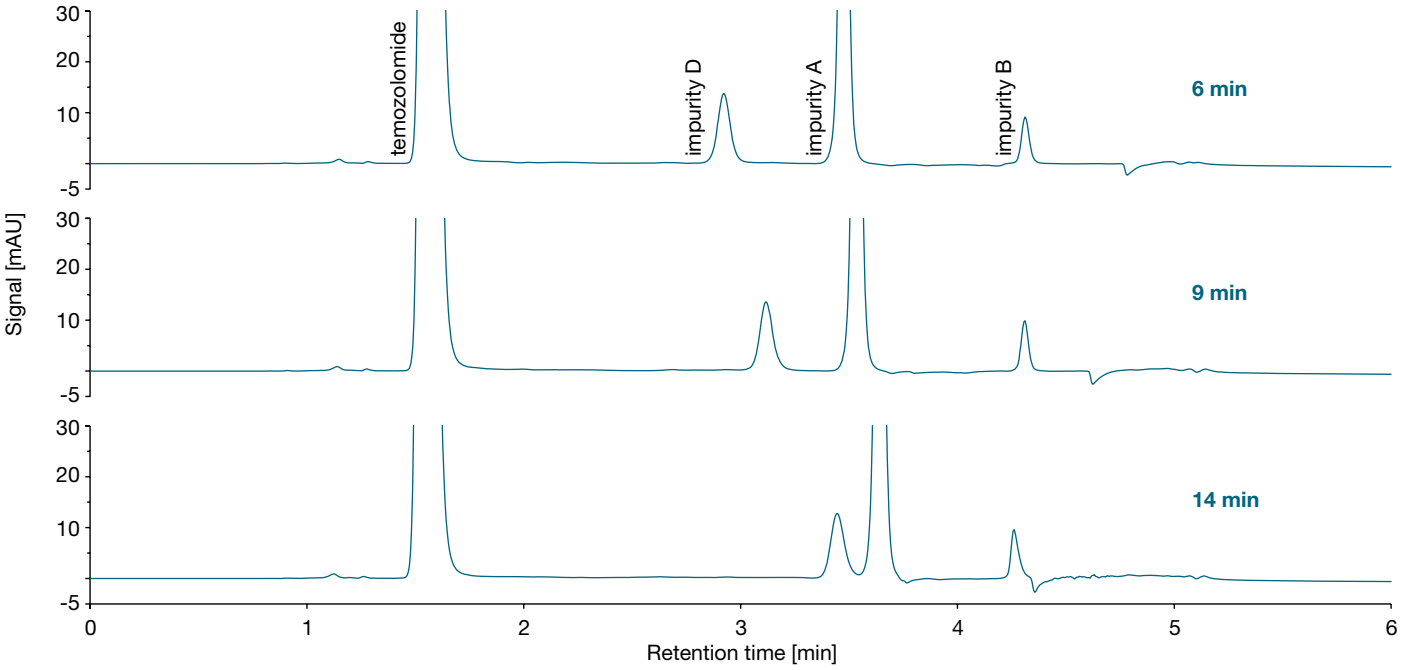

Figure 3. Selectivity changes of the HILIC method dependent on equilibration time (6, 9, and 14 min), shown for sample IV

Transfer

For best comparability, the transfer of the method from the 1260 Infinity system to the Vanquish Core HPLC system was conducted with the same column, aliquots of the same sample, and the same mobile phase batch to exclude non-instrumental effects on the transfer. Eight repeated injections of sample IV were executed with each system. Figure 4 displays the comparison of the chromatograms obtained with each system.

Very similar chromatograms were generated by the 1260 Infinity and Vanquish Core instruments, implying a very similar chromatographic performance. This can also be seen from Figure 5, which demonstrates a straightforward and successful method transfer. Retention times differed by less than 0.1 min and 3.5% from system to system. Both instruments provided repeatable retention times (t_R) and peak areas, expressed in low relative standard deviations (RSD). RSD in t_R is below 0.08% for each peak and system,

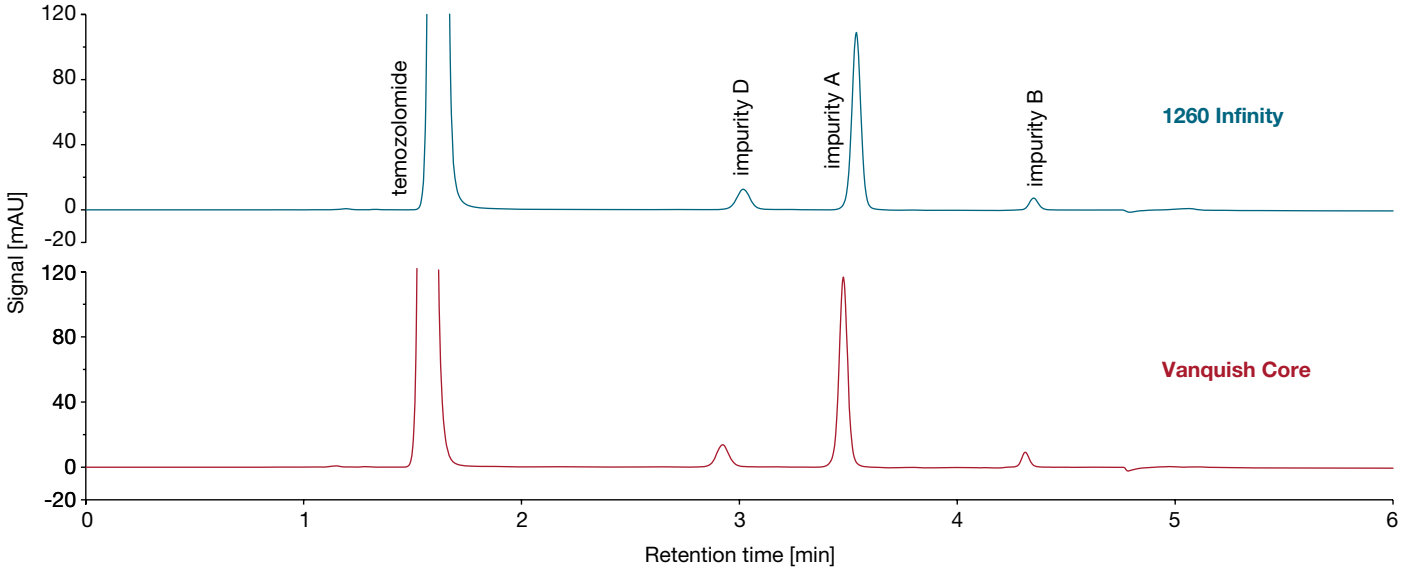

Figure 4. Transfer from 1260 Infinity system to Vanquish Core system

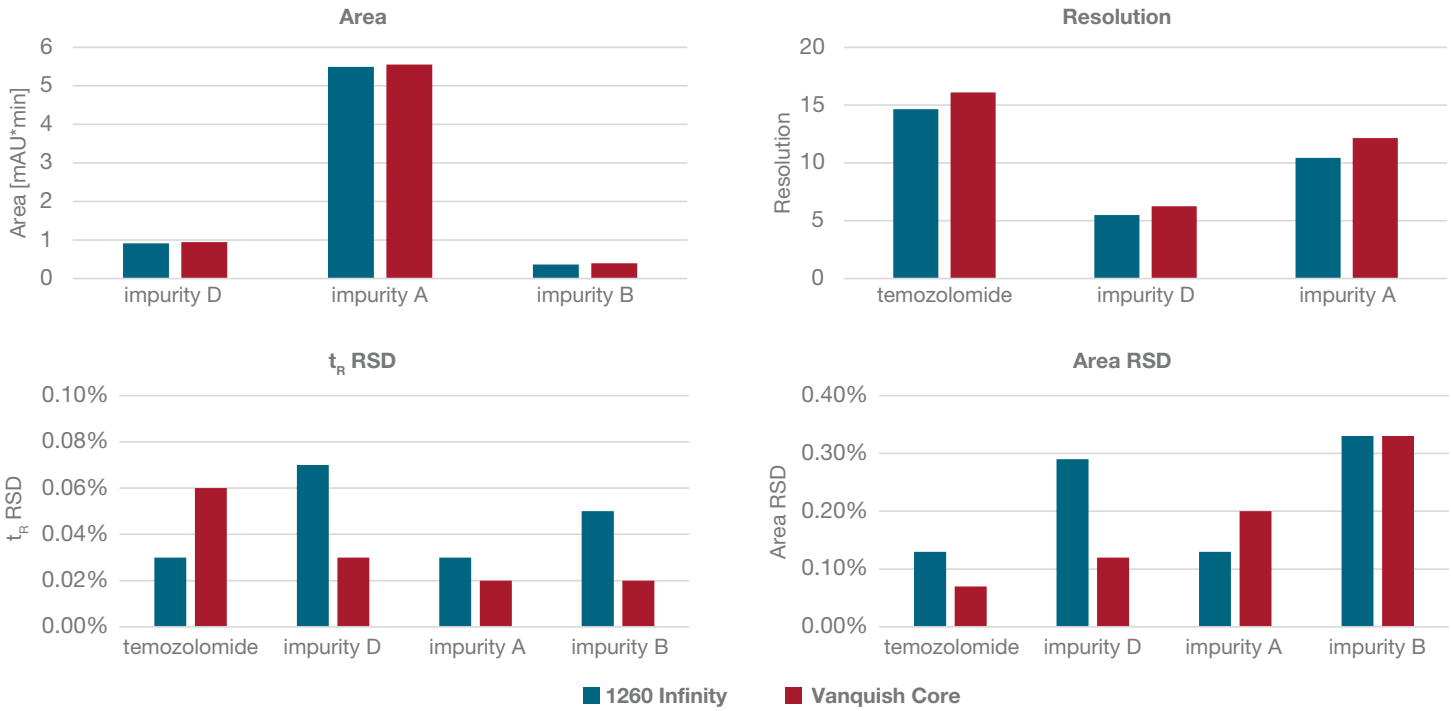

Figure 5. Chromatographic results and repeatability with 1260 Infinity and Vanquish Core systems

but overall better with the Vanquish Core system, while area RSD is below 0.4% and equivalent with both systems. The peak resolution is increased with the Vanquish Core system due to smaller peak widths (see appendix and Figure 8). The absolute peak areas of the impurities were in excellent accordance, for both systems. Areas of the API peaks differed distinctly, due to a difference in the detector response outside their linear range. The temozolomide peak considerably exceeded the specified linear range of both detectors (each 2500 mAU) as shown in Figure 6, which is common when APIs are overloaded to ensure the sensitive detection of low abundant impurities. Relative peak areas of impurities, referring to the API area, are affected by the mentioned response difference, and thus their use cannot be recommended when peaks overload detector capacities.

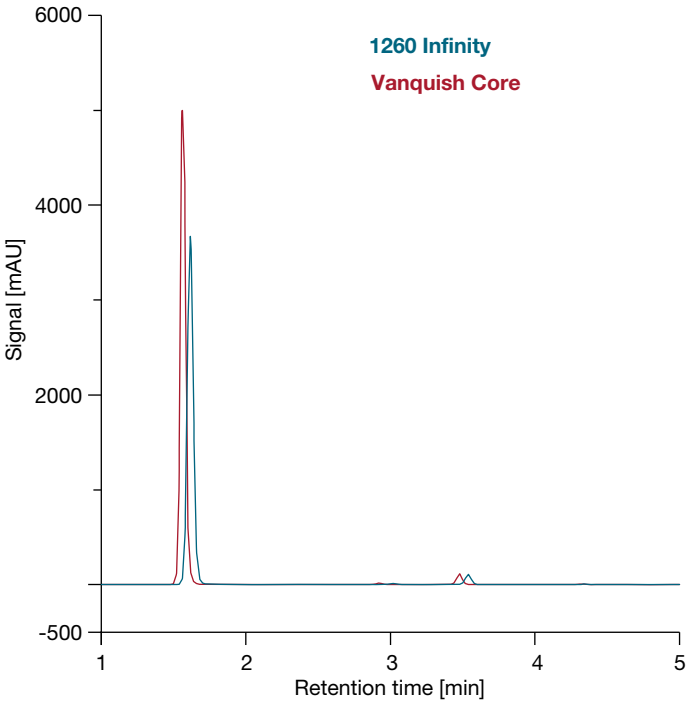

Figure 6. Unzoomed comparison of chromatograms obtained by 1260 Infinity and Vanquish Core instruments. While the temozolomide peak exceeds the linear range of both detectors, the dynamic response of the Vanquish Core VWD was distinctly higher than that of the 1260 Infinity VWD.

Conclusion

- A gradient HILIC method was set up for the impurity analysis of temozolomide with a run time of 12 min, giving reproducible results by applying the concept of repeatable partial equilibration.
- The transfer of the method from an Agilent 1260 Infinity LC system to a Thermo Scientific Vanquish Core HPLC system was a straightforward implementation, providing equivalent results with slightly more repeatable retention times and narrower peaks with the Vanquish Core system.
- Small offsets of absolute retention times could not be compensated by gradient delay volume adaption due to the effect on the partial equilibration of the column. Instead the root cause was found to be a difference in the extra column volumes of the systems (see Appendix).

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Appendix: Understanding residual retention time offsets

As mentioned above, the observed t_R deviations between the systems were small and did not compromise the success of the method transfer. A direct overlay is depicted in Figure 7A, showing slightly smaller t_Rs for each peak with the Vanquish Core system. Such t_R deviations may have several root causes like differences in the gradient delay volume (GDV) of the systems (volume between point of mobile phase mixing and column head), differences in the column thermostatting, differences in the extra-column volume (ECV, volume between points of injection and detection, excluding the column), or others. If a closer match of t_Rs is required, e.g., due to specified transfer limits, compensation of such instrumentational differences must be evaluated.

Usually, the first step is to vary the GDV, which can be easily implemented with the Vanquish Core instrument as it is equipped with a tunable idle volume of the autosamplers' metering device (0–230 µL and can be further increased by the Vanquish Method Transfer Kit to 430 µL).[2,3] However, in the current HILIC application, an increase of the GDV by moving the idle volume from default 25 µL to 100 µL did not result in a compensation of the t_R deviations but an unexpected reaction of t_Rs seen in Figure 7B with earlier elution of impurities D and A instead of later elution. This behavior can be explained by the phenomenon of partial equilibration of the HILIC column observed in the first section (Figure 3). With constant equilibration time but changing GDV, the column is in a slightly different equilibration state at the time of injection, which slightly affects the column selectivity. With much

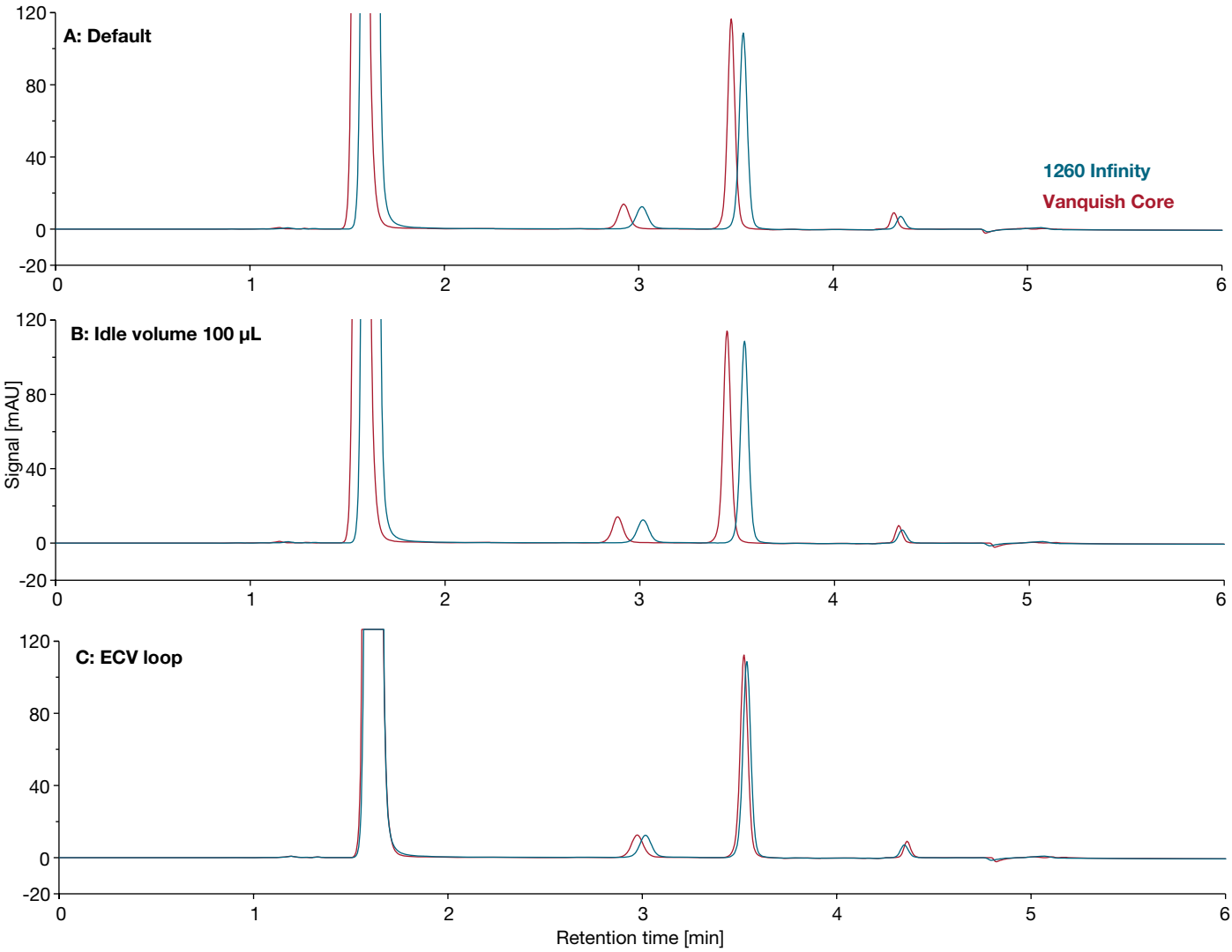

Figure 7. Overlay of chromatograms obtained by the 1260 Infinity instrument and the Vanquish Core instrument A) in default state (idle volume 25 µL); B) with idle volume set to 100 µL; C) with added ECV by 46 µL loop installed between injector and column. While the GDV adjustment could not compensate the t_R offset due to the partial column equilibration, the volume loop resulted in a closer match of ECVs.

longer equilibration times, the t_Rs increased as expected with increasing the idle volume (data not shown). But as the method was chosen to be as short as possible, GDV adaption was not the proper tool to compensate t_R offsets in the current method transfer.

Instead, the fact that the deviations were relatively constant throughout the chromatogram and affected each peak, even the early eluting temozolomide, pointed to a difference in the ECV of the systems. The assumption was confirmed by two experiments: 1) a direct flow injection of a caffeine standard in a water flow of 1 mL/min in both systems without a column and 2) by the injection of a dead volume marker (anthracene) with the HILIC method applied. The calculated t_R differences between the systems were corresponding to an ECV difference of 45 µL for the caffeine injection (t_R 0.088 min vs. 0.043 min) and 47 µL for the anthracene injection (t_R 1.120 min vs. 1.167 min).

The ECV of an LC instrument is affected by contributions from tubing, connectors, fittings, and detector flow cell. In general, low ECVs are related to more efficient chromatography due to minimized dispersion of peak bands. The installation of an additional volume loop (P/N 6036.2200), corresponding to 46 µL, between the injector and the column in the Vanquish Core system resulted in a distinctly improved match of all t_Rs regarding the 1260 Infinity system with offsets not higher than 0.05 min as seen in Figure 7C and Figure 8. Although the loop technically also adds up to the GDV, its effect was different from changing the idle volume since the sample was also traveling through the added volume. The downside of that approach was an increase of peak widths compared to the default setup due to an increased peak dispersion by the loop (Figure 8). Thus, the installation of extra system volume is only recommended if other strong benefits apply, e.g., proper pre-column mixing of strong sample plugs and mobile phase.

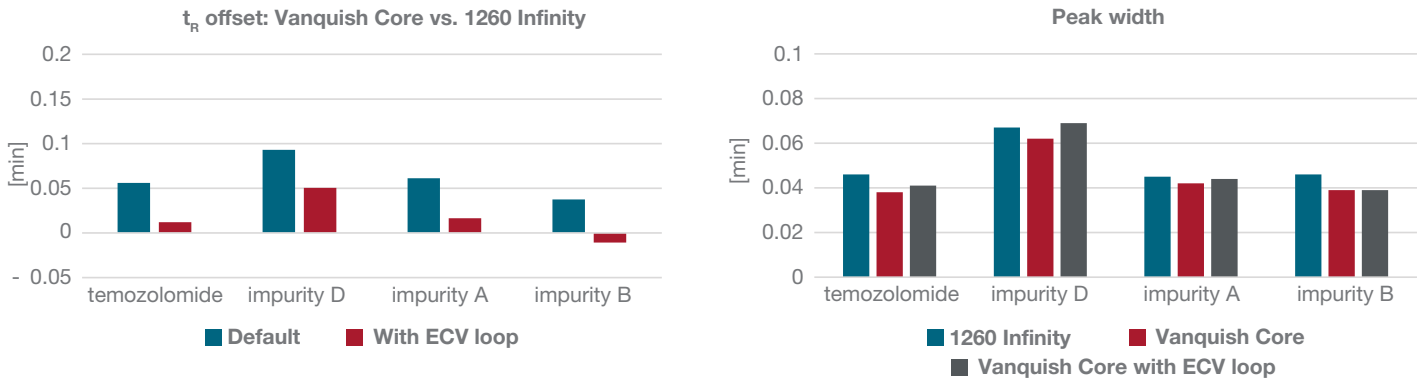

Figure 8. Effect of added ECV on the method transfer from the 1260 Infinity to the Vanquish Core system regarding t_R offset and peak widths (at half height)

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